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Semiconductor technology in protein kinase research and drug discovery: sensing a revolution.

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Abstract

Since the discovery of ‘protein kinase activity’ in 1954, close to 600 kinases have been discovered that play a critical role in cell physiology. In several pathological conditions, aberrant protein kinase activity leads to abnormal cell and tissue physiology. Therefore, protein kinase inhibitors are investigated as potential treatments for several diseases, including dementia, diabetes, cancer, autoimmune and cardiovascular disease. Modern

semiconductor technology has recently been applied to accelerate the discovery of novel protein kinase inhibitors that may become the standard of care drugs of tomorrow. Here we describe current techniques and novel applications of semiconductor technologies in protein kinase inhibitor drug discovery.

Teaser

Semiconductor technology opens new frontiers in the detection of biochemical reactions *in vitro*. Here, we describe its application to the field of protein phosphorylation research and kinase inhibitor drug discovery.

Introduction

The therapeutic need for novel and efficacious treatments remains unmet for several human diseases. In addition, even when effective treatments are available, the onset of drug resistance or the occurrence of side effects associated with prolonged drug use requires the discovery of novel drugs [1]. The acceleration of the drug discovery cycle remains a priority in several biomedical fields and will depend on the availability of new tools for drug screening in academia and industry. Protein kinase inhibitors are one of the most important target groups for drug development [2]. Nonetheless, only a small portion of human protein kinases can be effectively modulated using specific inhibitors [3]. To date, more than 400 diseases are reported to be caused either directly or indirectly by deregulation or mutation of protein kinases [4], and over 200 protein kinase genes map to disease loci or cancer amplicons [5]. The drug development opportunities are enormous, but the rate of discovery and approval of new protein kinase-targeting drugs remains low [6, 7]. Part of the problem resides in the *in vitro*

assays used for the primary screening of compounds that inhibit or modulate protein kinase activity.

The most common techniques used to investigate protein kinase activity are kinase assay sthat use 32 -phosphorus (^{32}P) labelling of peptides designed to mimic the kinases target sequences [8] or phosphor-specific immunodetection assays (e.g. ELISA, immunoblots or flow cytometry), which rely on antibodies selectively recognising their targets when phosphorylated [9], or spectroscopic techniques with nanoparticles acting as markers for phosphorylation [10]. Mass spectrometry-based detection of protein phosphorylation or phosphoproteomics has also become a very important technique in protein kinase investigations [11]. Despite recent developments facilitating their use for basic research and target identification (e.g. [12]), none of the above techniques has the high throughput necessary for primary drug screening. For drug discovery purposes, binding assays are utilised to test the ability of putative compounds to inhibit the interaction of protein kinases with their protein substrates [13] or their binding of ATP [14]. Fluorescence-based assays have also become widely used for the screening of large libraries of compounds and development of new drugs [15]. Fluorescent biosensors for protein kinase studies entail a biochemical moiety that recognizes the target kinase in its active state (e.g. a substrate or binding domain), which is coupled to a fluorescent probe and therefore transduces kinase activation into a measurable signal. Both traditional binding or fluorescence biosensor-based assays have an important limitation: they identify molecules targeting the specific molecular event that they measure (i.e. binding to specific ligand or binding of ATP). This leads to the loss as false negatives of compounds targeting other aspects of the enzymatic reaction, which is responsible for a weak correlation between binding/fluorescence-based screenings and

kinase activity assay results [16]. Screening results based on binding assays can therefore be misinterpreted and promising protein kinase inhibitors can be missed. Similarly, drug screenings based on the detection of endpoint functional responses (e.g. cell apoptosis, proliferation or metabolic throughput), have the important limitation of identifying compounds affecting the event providing the readout signal, rather than directly binding and modulating protein kinases.

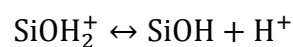
We have recently described a novel method based on the use of an electrolyte-insulator-semiconductor (EIS) for the detection of the protein phosphorylation reaction [17-19]. This method has several advantages over existing methods, including: 1) identification of compounds interfering with different aspects of the catalytic mechanism of protein kinases (rather than just interfering with ATP- or substrate-binding); 2) detection of phosphorylation in real time and monitoring the rate of phosphorylation reactions, therefore allowing kinetics studies (rather than relying on the end-point detection of reaction products – i.e. phosphorylated substrates by ^{32}P incorporation and phospho-dependent immunodetection); 3) high throughput and potential for upscaling; 4) low cost.

Semiconductor technology applied to biology and drug discovery

Recent developments in the semiconductor technology may open a new era for drug discovery research, characterised by assays capable of screening thousands of inhibitors of kinases in a single run. The most cost-effective way to apply semiconductor technology to the realm of chemical or biological sensing is by using field effect devices (FEDs) such as ion-sensitive field effect transistor (ISFETs) and EIS.

ISFETs measure the changes of potential on the interface between a dielectric material and an electrolyte, typically as a result of the adsorption of ions on the surface of the material. Several dielectric materials used in the semiconductor industry – such as, for example, silicon nitride and aluminium oxide – are pH sensitive (*i.e.* adsorb H^+ ions). A large number of intra/inter cellular reactions involve charge transfer or release of protons. Upon surface immobilization of substrates or enzymes, the release of protons or the minute changes in electrical charge associated with chemical reactions can therefore be measured. A commonly used pH sensitive material is silicon nitride, which in our recent studies has been employed to measure the release of protons associated with protein phosphorylation [17-19]. The surface charge of the silicon nitride can be measured by integrating it with a semiconductor in form of an ISFET or EIS sensor.

The detection mechanism of EIS is similar to the operation of ISFETs. The difference lies in the fact that the EIS is a two-terminal capacitor structure, while the ISFET has extra contacts on doped regions of the semiconductor to operate as a three-terminal transistor. The adsorption of ions on the surface of the dielectric material creates an electric field across the dielectric material, inducing changes in the underlying semiconductor. The mechanism can be explained by site binding theory, which relates the solid-liquid interface potential to local pH changes in the solution [20]. In the case of silicon nitride, at the dielectric material/solution interface, Si_3N_4 presents active sites in the form of neutral surface hydroxyl (SiOH) groups, which capture H^+ ions released by chemical reactions until thermodynamic equilibrium is established, as described by the following reaction:



After the hydrogen ion is captured by the silicon nitride, the surface potential of the structure changes. If this structure acts as the gate of the transistor, gate to source voltage of the transistor would also change. Equation 1.1 below shows the dependence of the semiconductor surface potential (ϕ_s) on the hydrogen ion concentration:

$$\phi_s = -\frac{C_1}{C_h}(E_1d_2 + E_2d_2) + \phi_d + \frac{qN_{sil}}{C_h} \left\{ \frac{[H_s^+]^2 - K_+K_-}{[H_s^+]^2 + K_+[H_s^+] + K_+K_-} \right\} + \frac{qN_{sil}}{C_h} \left\{ \frac{[H_s^+]^2 - K_+K_-}{[H_s^+] + K_{N+}} \right\} \quad (1.1)$$

where C_1 is the accumulation capacitance due to the two dielectric layers, namely Si_3N_4 and SiO_2 in the structure that has been used. $E_{1,2}$ and $d_{1,2}$ denote the electric field and thickness of each dielectric layer. C_h is the Helmholtz layer capacitance per unit area, N_{sil} is the number of silanol sites per unit area and $K_{+/-}$ is the dissociation constant for the chemical reaction at the insulator. It is apparent from Equation 1.1 that a highly sensitive pH sensor would require a relatively large value of N_{sil} . The presence of two dielectric layers increases the pH sensitivity of the surface. With two dielectric layers, as compared with a single insulator structure with the same concentration of H^+ ions on the surface, higher surface potential is achieved. This increase in surface potential decreases the threshold seen by the channel at the transistor gate. As a result, at a given voltage, the depletion charge increases at higher concentration of H^+ ions.

The surface potential (ϕ_s) on the insulator of EIS structure is measured by capacitance voltage characterisation. In this technique, a dc voltage superimposed with a small ac signal is applied across the EIS structure and the capacitance of the structure is measured. There are three distinct regions of the curve: 1) inversion 2) depletion and 3) accumulation. The inversion region is formed with minority charge carriers and the

accumulation region is formed by the majority carriers in the bulk substrate. When a low frequency ac signal (less than 100 Hz) is applied, the saturation values of inversion and accumulation coincide with each other. On increasing the frequency, the inversion capacitance decreases because at high frequencies (greater than 1 kHz) the generation rate is not fast enough to allow the formation of minority carriers at the insulator-semiconductor interface. However, the accumulation and depletion capacitances do not vary with the change in the frequency. The accumulation capacitance is the intrinsic capacitance of the structure (due to the thickness and dielectric constant of the insulator), which is independent on any charges on the surface. Any charges on the surface, for instance protein charge or a pH change on the surface, are reflected on the voltage required to induce depletion (the threshold voltage).

ISFETS have been used extensively for decades for pH sensing applications (with variable degrees of commercial success). However, despite their amenability for biochemical sensing the only ISFET based high-throughput technology commercially released so far is Ion Torrent whole genome sequencing system.. Currently, this technology appears to be the cheapest and most time effective solution for whole-genome sequencing. FEDs can function as micro-pH sensors and measure the release of protons associated with chemical reactions. There is a large number of chemical reactions in the human body that involve release of protons that could be readily detected by both ISFETs and EISs, including protein phosphorylation.

The major advantage of using FED-based devices is their high throughput capabilities. The potential of very large scale integration technology (VLSI) to form an array of FEDs to monitor biocatalytic transformation has been exploited by Ion Torrent on their DNA sequencing chip [20]. This technology allows whole genome sequencing on a chip

by detection of protons released by the elongation of DNA primer molecules during a polymerase chain reaction (PCR). The release of protons is measured by an array of ISFETs. Similarly, the release of proton associated with phosphorylation of proteins or other biochemical reactions can be easily measured by FEDs. Such a system would enable the analysis of different biochemical reactions and the screening of various inhibitors (drugs) in an effective and convenient manner [22]. Therefore, its application in the development of miniaturised drug discovery platforms is promising and deserves attention.

Protein phosphorylation on EIS Sensors

EIS sensors have become established in the biosensing community as powerful tools to detect pH changes in highly resolved temporal and spatial patterns [23, 24]. In the context of protein phosphorylation, there have been previous studies focusing on the detection of the change in substrate protein electrical charge rather than on the release of protons in the protein kinase reaction. Freeman et al. were the first to study the degree of gate surface charging detectors due to kinase activity [25]. They used an aluminium oxide gated ISFET to follow the phosphorylation of proteins. This system allowed monitoring of both phosphorylation and dephosphorylation in real-time, leading to the estimation of phosphorylation reaction kinetics. Electrochemical detection of protein kinase activity using FEDs has been demonstrated in several relevant studies using different semiconductive materials, such as silicon [26], indium tin oxide (ITO) [27] and boron-doped diamond [28]. In other studies, different reagents were utilised to enhance the detection of the protein kinase reaction, including TiO₂/silver nanoparticles [29] and Carbon nanotubes (CNTs) [30]. The same authors also demonstrated the use of

gold nanoparticles combined with semiconductor technology to obtain the electrochemical detection of the enzymatic reaction catalysed by tyrosine protein kinases [31].

In our recent work, we demonstrated that a semiconductor-based EIS can be applied to dual detection of proton release and surface charge change associated with the phosphorylation of surface-absorbed proteins [17-19]. Figure 1 shows a detection scheme for dual mode biosensing employed by Bhalla *et al.* [17]. In this work the authors used Si_3N_4 -based EIS structures as well as gold-insulator-semiconductor capacitor structures for the detection of pH and electrical charge changes associated with phosphorylation of proteins, respectively. In both methods, a conventional three-electrode electrochemical setup was employed with an Ag/AgCl reference electrode immersed in the electrolyte via a salt bridge, used to apply the gate voltage, and a platinum (Pt) counter electrode. The Capacitance-Voltage (CV) characteristics of the sensor were monitored to detect the kinase activity.

Upon kinase-mediated protein phosphorylation, each amino acid undergoing phosphorylation releases a proton that is adsorbed onto the Si_3N_4 surface. As a result, the threshold potential of the structure decreases and a change in potential is observed. The longer the reaction with kinase/ATP is left to progress, the higher the V_g changes that are achieved. Our studies demonstrated the use of EIS to study phosphorylation of myelin basic protein (MBP) by PKC- α in the presence of a phosphate source (ATP /ATP-S) [17-19]. The phosphorylation reaction and its inhibition could be detected, as the phosphorylation reaction was associated with significantly higher signal (37 mV) compared to the inhibited reaction (5 mV).

Similar results were obtained when reaction was performed using ATP-S instead of ATP. The use of ATP-S for phosphorylation adds a thiol group to the protein upon phosphorylation. The thiophosphorylation of proteins allowed the use of AuNPs (16 nm mean size), wherein the affinity between the thiol groups and Au resulted in the binding of AuNPs. As a consequence, the phosphorylated protein was labelled with AuNPs which enabled its detection by localised surface plasmon resonance (LSPR), alongside pH changes on the EIS substrate. Upon thiophosphorylation, a red-shift of 31 nm in the absorption spectrum of silicon nitride was observed. The reaction performed in the presence of kinase inhibitor revealed less than 10 nm of shift, confirming that the large redshift in the wavelength of thiophosphorylated sample was due to the covalent attachment of AuNPs to the sulphide group present on the γ -phosphate group transferred from ATP-S to the MBP (Figure 2).

The findings of Bhalla et al. [17] strongly argue for the use of electrical and optical detection as orthogonal tool to detect kinase activity in a single experiment. Complementary characterisation techniques are often required to characterize and validate biomolecular interactions. The authors work shows two orthogonal techniques being used on a single platform for fast and miniaturised biosensing. Each technique provides different information, which can then be correlated to standard curves of analyte concentration. In the case of the presented dual LSPR and EIS semiconductor system, EIS provides real time information on phosphorylation kinetics, while LSPR confirms the reaction completion. In addition, because LSPR readouts inherently change upon nanoparticle binding to the substrate, in cases of lower phosphorylation activity, where it would be difficult to detect the phosphorylation reaction using only an EIS system, LSPR facilitates the detection of the reaction and its completion.

Conclusions and Outlook

Semiconductor EIS devices display remarkable performances in the detection of protein phosphorylation. Development of arrays of such structures with microfluidics can be utilised for the development of an automated device that can be used to screen novel inhibitors of kinases. Using complementary metal oxide semiconductor (CMOS) and VLSI technology, it could be possible to design a high throughput platform for screening inhibitors of protein kinases.

This technique has so far been tested using recombinantly expressed active protein kinases and their peptide substrates. Although the generation of substrates by customised peptide synthesis is easily achievable and inexpensive, the availability of catalytically active protein kinases may represent a bottle neck for the implementation of this approach. On this respect, it must be noted that an increasing number of protein kinases are commercially available as catalytically active enzymes through a number of specialised manufacturers and suppliers. Where the desired protein kinases is not readily available, modern molecular biology and expression systems in eukaryotic cells are readily available for cloning and expression of protein kinases as catalytically active enzymes [32, 33]. In addition, although this option has not been experimentally tested, further development may enable the use of cell lysates or tissue extracts as sources of active kinases. This approach would simplify the technique, reduce the costs and allow the investigation of disease states for which the responsible kinase is yet unknown.

A final advantage of the use of semiconductors for drug discovery in particular and biosensing in general is their amenability to technology multiplexing. By combining several orthogonal detection technologies on a single platform, a robust system for

compound screening could be designed. The integration of multiple technologies on a single biosensor is theoretically possible and could allow the parallel study of more than one biochemical parameter at once, such as association/dissociation constant, real-time kinetics, rate of product formation, etc. This can provide important information about the characteristics of drugs under development in a convenient manner.

Figure legends

Figure 1 (A) Immobilised protein on silicon nitride surface. (B) Upon thiophosphorylation there is a release of proton (which allows EIS-dependent monitoring of the reaction) and transfer of γ phosphate from ATP-S. (C) Covalent attachment of AuNPs to the sulphide group on thiophosphorylated protein. (D) LSPR detection mechanism

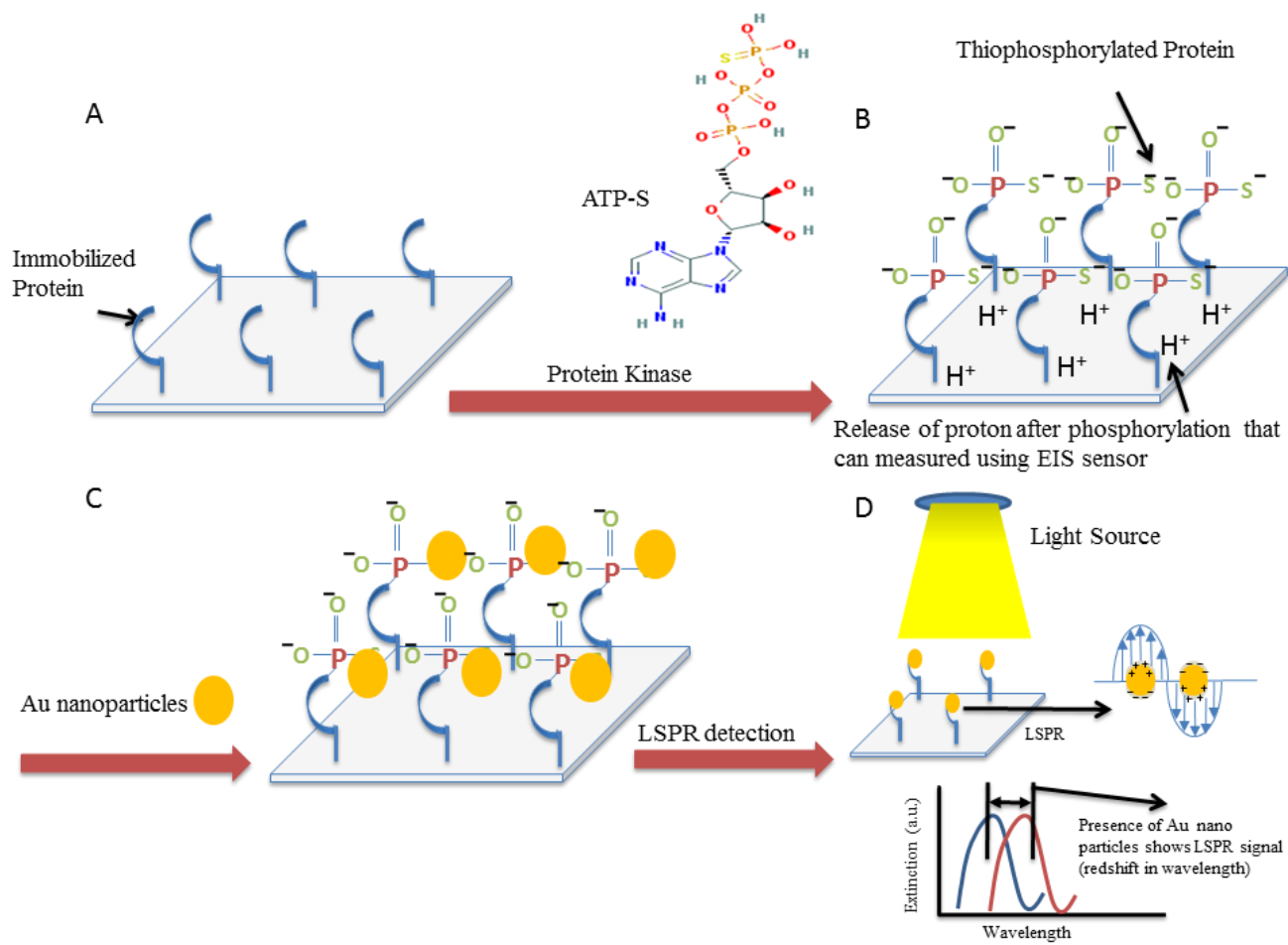
Figure 2: (a) LSPR spectra in the absorption mode for samples thiophosphorylated and controls (without kinase activator, ATP phosphorylation, and in the presence of an inhibitor), as well as for the Si_3N_4 background. (b) Kinase activity detected by measuring the release of proton associated with protein phosphorylation for the same samples shown in 2a.

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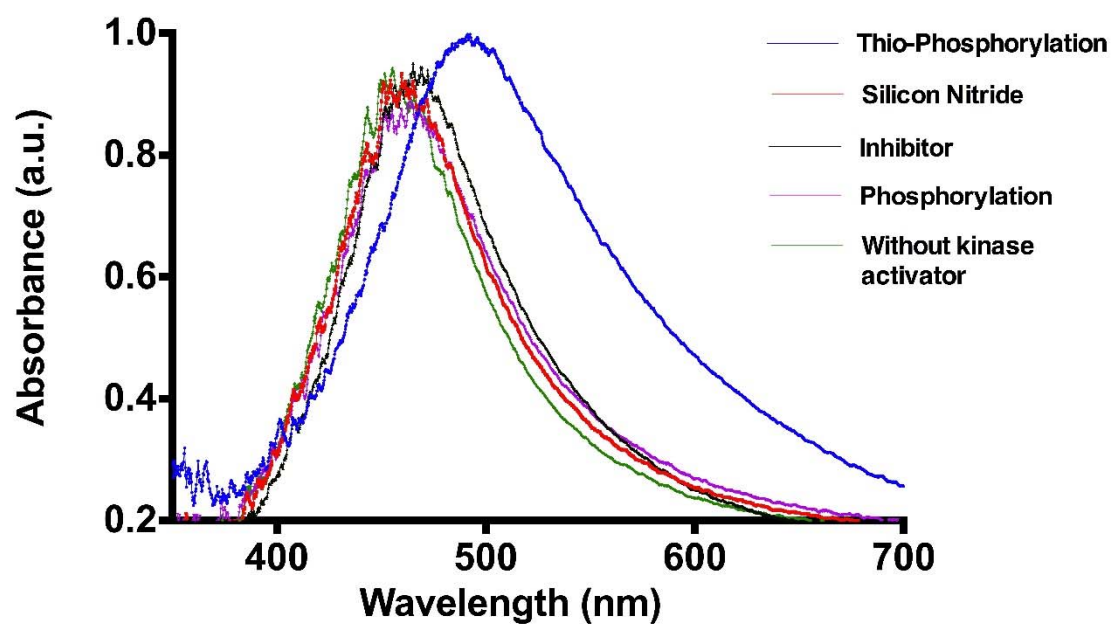
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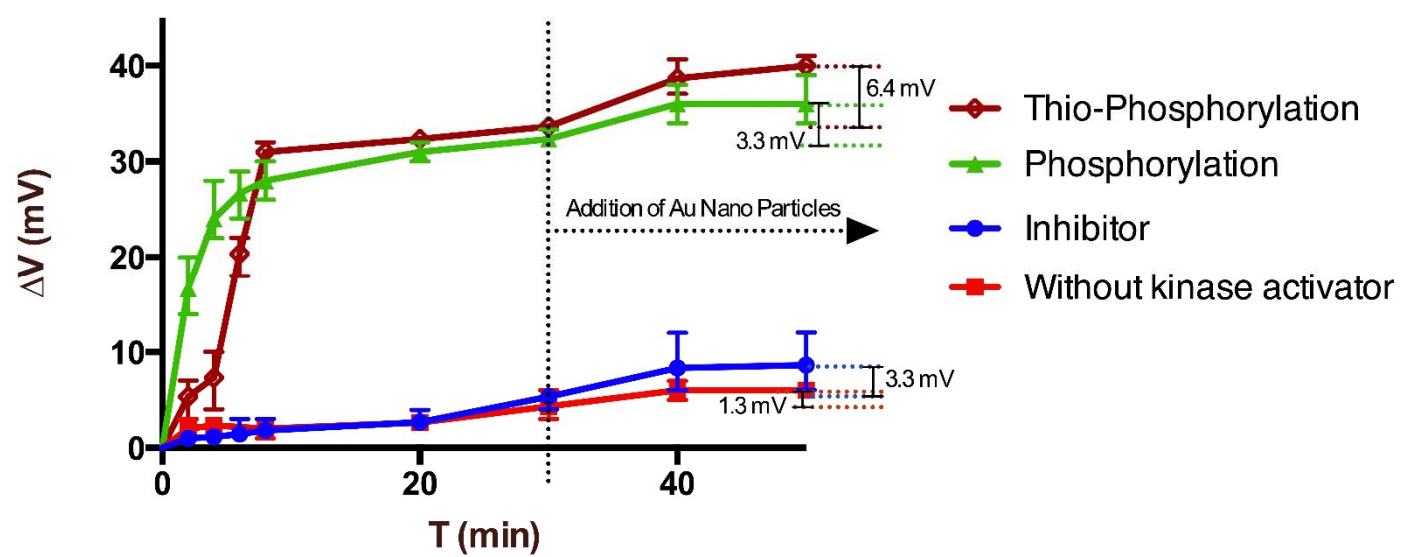
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A



B



Research Highlights:

- We developed a new dual detection technology for protein phosphorylation
- It combines electrolyte-insulator-semiconductor and localised surface plasmon resonance
- It does not require radioactive labelling or phospho-specific antibodies
- It offers the possibility to follow the phosphorylation reaction in real time
- It is a formidable tool for kinase inhibitor drug discovery